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(57) Abstract

In general, the invention features a mutant pore–forming toxin, wherein the toxin comprises a mutation in an amino acid that forms the transmembrane pore of said toxin. Also included is substantially pure nucleic acid that encodes the mutant pore–forming toxin, as well as methods of decreasing toxicity of a pore–forming toxin by administering a mutant pore–forming toxin in a dose sufficient to inhibit translocation of a pore–forming toxin.

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INHIBITION OF TOXIN TRANSLOCATION

Background of the Invention

The invention relates generally to the prevention and treatment of toxicity due to pore-forming toxins, particularly A-B toxins.

Many bacterial protein toxins enzymatically modify specific intracellular constituents of eukaryotic target cells. Intracellularly acting bacterial toxins must cross a membrane barrier of host cells to reach their cytosolic targets. Most intracellularly acting toxins may be classified as A-B toxins, where the B moiety binds to the surface of the host cell and translocates the enzymatic A moiety into the cytosol (Gill, D. M., in Bacterial Toxins and Cell Membranes, Jeljaszewicz, J., & Wadstrom, T., Eds., pp 291-322, Academic Press, New York, 1978). In many of these toxins, membrane translocation of the A moiety involves insertion of the B moiety into the host membrane, resulting in the formation of an ion-conducting pore. X-ray crystal structures have been solved for the water soluble forms of two pore-forming A-B toxins and also of other toxins that function simply by forming pores at the cell surface (Parker, M. W. et al., Nature 337:93-96, 1989; Li, J. D. et al., Nature 353:815-21, 1991; Choe, S. et al., Nature 357:216-22, 1992; Parker, M. W. et al., Nature 367, 292-95, 1994; Petosa, C. et al., Nature 385;833-38,1997). The crystal structure of one pore-forming toxin has been solved in its membrane-inserted form (Song, L. et al., Science 274:1859-66, 1996). Membrane insertion by these water soluble proteins involves conformational changes to expose or generate new surfaces which can penetrate hydrophobic membrane barriers. In the case of pore-forming A-B toxins, it is unclear whether the pore formed by the B moiety serves as a conduit for translocation or is a by-product of membrane penetration.

Many bacterial toxins, such as diphtheria toxin, contain both moieties in a single polypeptide. In contrast, the A and B moieties of binary toxins are contained in

separate proteins which self-assemble at the mammalian cell surface. An example of a binary toxin is anthrax. In anthrax toxin, the B moiety, Protective Antigen (PA), mediates the translocation of two alternative A moieties, Edema Factor (EF) and Lethal Factor (LF), into the cytosol. Under the current model for anthrax intoxication, PA first binds to a ubiquitous cell surface receptor (Escuyer, V., & Collier, R. J., Infect Immun 59:3381-86, 1991) and is then cleaved by furin or a furin-like protease (Klimpel, K. R. et al., Proc Natl Acad Sci U S A 89:10277-81, 1992). Proteolytic removal of the N-terminal 20 kDa segment enables the remaining receptor-bound 63 kDa fragment (PA_{63}) to form a heptameric prepore (Milne, J. C. et al., J. Biol. Chem. 269:20607-612, 1994) and to bind EF or LF (Leppla, S. H. et al., in Bacterial Protein Toxins, Ferenbach, Ed., pp 111-112, Gustav Fisher, Stuttgart, 1988). The entire complex is then trafficked to the endosome, where the low pH environment induces the PA_{63} prepore to insert into the membrane and translocate EF and LF to the cytosol (Gordon, V. M. et al., Infect Immun 56:1066-69, 1988; Friedlander, A. M., J Biol Chem 261:7123-26, 1986). In the cytosol, both EF and LF catalyze reactions that have toxic effects (Leppla, S. H., Proc Natl Acad Sci U S A 79:3162-66, 1982; Hanna, P. C. et al., Proc Natl Acad Sci U S A 90:10198-201, 1993).

Insertion of the PA_{63} heptamer into the endosomal membrane is believed to mediate translocation of EF and LF. Elucidation of the insertion mechanism of pore-forming toxins would allow prevention of the translocation process, which, in turn, could provide an effective means for reducing toxicity associate with these viruses.

Summary of the Invention

In a first aspect, the invention features a purified protein that includes a mutant pore-forming toxin, wherein the toxin comprises a mutation of an amino acid that forms the transmembrane pore of the toxin.

In preferred embodiments, the mutation is a missense mutation or a deletion, is genetically engineered, and reduces the toxicity of a pore-forming toxin. In other

embodiments, the mutation inhibits the translocation of the wild-type pore-forming toxin across the transmembrane pore, and decreases conductance measured at the transmembrane pore.

Additional embodiments of this aspect include a mutant pore-forming toxin that is a mutant B-moiety of an A-B toxin, a mutant B-moiety of a binary toxin, preferably, the B-moiety is an anthrax toxin protective antigen (PA), or its C-terminal 63 kDa tryptic fragment (PA_{63}).

In preferred embodiments, the mutation is located in the amino acid sequence that includes the transmembrane pore of the pore-forming toxin, preferably, at the hydrophilic face of a transmembrane pore, preferably, in the D2L2 loop, more preferably, in the D2L2 loop of PA or PA_{63}, and, most preferably, in amino acid residue E302, H304, N306, E308, H310, S312, F313, F314, D315, G317, S319, S321, G323, or S325 of the D2L2 loop.

Additional embodiments provide that the mutant pore-forming toxin is streptolysin O, α-toxin, A-B toxin (staph-α or thiol-activated), or binary toxin (diptheria or exotoxin A), preferably, the mutant pore-forming toxin is anthrax.

In a second aspect of the invention, the invention features a substantially pure nucleic acid sequence encoding the amino acid sequence of the mutant pore-forming toxin protein, wherein the toxin has a mutation in an amino acid that forms the transmembrane pore of said toxin.

In preferred embodiments, the mutation in the pore-forming protein is a missense mutation or a deletion, is genetically engineered, and reduces the toxic response to a pore-forming toxin. In other embodiments, the mutation in the pore-forming toxin inhibits the translocation of the wild-type pore-forming toxin across the transmembrane pore, and decreases conductance measured at the transmembrane pore.

Additional embodiments of this aspect include a nucleic acid encoding a mutant pore-forming toxin that is a mutant B-moiety of an A-B toxin, a mutant B-moiety of a binary toxin, preferably, the B-moiety is an anthrax toxin protective

antigen (PA), or its C-terminal 63 kDa tryptic fragment (PA_{63}).

In preferred embodiments, the mutation is located in the amino acid sequence that includes the transmembrane pore of the pore-forming toxin, preferably, at the hydrophilic face of a transmembrane pore, preferably, in the D2L2 loop, more preferably, in the D2L2 loop of PA or PA_{63}, and, most preferably, in amino acid residue E302, H304, N306, E308, H310, S312, F313, F314, D315, G317, S319, S321, G323, or S325 of the D2L2 loop.

Additional embodiments provide that the nucleic acid encodes a mutant pore-forming toxin that is streptolysin O, α-toxin, A-B toxin (staph-α or thiol-activated), or binary toxin (diptheria or exotoxin A), preferably, the mutant pore-forming toxin is anthrax.

In a third aspect, the invention features a method of decreasing the toxic response to a pore-forming toxin, that includes administering a protein that is a mutant pore-forming toxin, wherein the toxin includes a mutation of an amino acid that forms the transmembrane pore of the toxin, in a dose sufficient to inhibit translocation of a pore-forming toxin.

In preferred embodiments of the third aspect, the mutant protein functions as a dominant mutant to inhibit translocation of a wild-type pore-forming toxin, the protein is administered prophylactically, and the protein generates antibody production directed against a wild-type sequence pore-forming toxin.

In preferred embodiments, the toxin's mutation is a missense mutation or a deletion, is genetically engineered, and reduces the toxic response to a pore-forming toxin. In other embodiments, the mutation inhibits the translocation of the wild-type pore-forming toxin across the transmembrane pore, and decreases conductance measured at the transmembrane pore.

Additional embodiments of this aspect include administering a mutant pore-forming toxin that is a mutant B-moiety of an A-B toxin, a mutant B-moiety of a binary toxin, preferably, the B-moiety is an anthrax toxin protective antigen (PA), or

its C-terminal 63 kDa tryptic fragment (PA_{63}).

In preferred embodiments, the mutation is located in the amino acid sequence that includes the transmembrane pore of the pore-forming toxin, preferably, at the hydrophilic face of a transmembrane pore, preferably, in the D2L2 loop, more preferably, in the D2L2 loop of PA or PA_{63}, and, most preferably, in amino acid residue E302, H304, N306, E308, H310, S312, F313, F314, D315, G317, S319, S321, G323, or S325 of the D2L2 loop.

Additional embodiments provide for administering mutant pore-forming toxin that is streptolysin O, α-toxin, A-B toxin (staph-α or thiol-activated), or binary toxin (diptheria or exotoxin A), preferably, the mutant pore-forming toxin is anthrax.

"Mutation" means an alteration in the nucleic acid sequence such that the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid alteration from the wild-type sequence. The mutation may, without limitation, be an insertion, deletion, frameshift mutation, or a missense mutation, or other modification which alters protein function as described herein.

"Dominant mutant" toxin means a mutant toxin which effectively inhibits the translocation of wild type toxin by forming transmembrane pore hybrid heptamers with the wild-type pore-forming toxin proteins and interfering with normal pore formation or translocation of toxin.

"Pore-forming toxin" means a toxin which traverses the membrane barrier through its transmembrane pore and enzymatically modifies specific intracellular substrates of a host cell.

"Pore-forming A-B toxin" means a pore-forming toxin with two functional moieties; one moiety (B) which forms a pore in a host cell barrier membrane, and the other (A) traverses the membrane barrier through the transmembrane pore and enzymatically modifies specific intracellular substrates of a host cell.

"Binary toxin" means a pore-forming A-B toxin in which the A and B moieties of the pore-forming toxin inhabit separate proteins, and interact during the intoxication

of host cells. An example of a binary toxin is anthrax toxin.

"B moiety" means the component of a pore-forming A-B toxin which binds a specific host cell-surface receptor, forms a transmembrane pore in the host cell membrane, binds the A moiety toxin, and functions to translocate the A moiety into the host cell cytoplasm.

"Protective antigen (PA)" means the anthrax toxin B moiety.

"PA_{63}" means the carboxy-terminal portion that results from proteolytic cleavage of a 20 kDa N-terminal segment from the PA polypeptide. PA_{63} forms a heptameric prepore and binds the two alternative A moieties, edema factor (EF) and lethal factor (LF). The entire complex is trafficked to the endosome, where PA_{63} inserts into the membrane, forms a transmembrane pore, and translocates EF and LF into the host cell cytoplasm.

"Transmembrane pore" means the β-barrel channel formed by alternating hydrophilic and hydrophobic residues of the pore-forming toxin protein such that the hydrophobic residues form an exterior membrane-contiguous surface of the barrel, and the hydrophilic residues face an aqueous lumen of a pore that spans across the host cell membrane.

"D2L2 loop" means the amphipathic loop which connects strands 2β2 and 2β3 of PA polypeptide and PA_{63} polypeptide as described herein.

"Decreased conductance measured at the transmembrane pore" means a reduction in conductance at the transmembrane pore of the toxin in response to derivatization of an amino acid residue on the hydrophilic face by a positively charged reagent, as measured by macroscopic or single channel analysis. Preferably, the positively charged reagent is methanethiosulfonate ethyl sulfate (MTS-ET). In addition, the decrease in conductance is preferably at least 20% relative to the conductance across the transmembrane pore of a wild-type pore-forming toxin in the presence of the positively charged reagent. More preferably, the decrease is at least 40%.

"Inhibit translocation" means a reduction in the process by which the toxin moiety of pore-forming toxin is transferred through the transmembrane pore into the host cell cytoplasm. This decrease in toxin translocation is positively correlated with, and could be predicted by, a decrease in conductance measured at the transmembrane pore in response to derivatization by a positively charged mutant (as previously described at page 9, line 14 to page 10, line 1). Preferably, the decrease in translocation results in decreased toxin transfer by at least 20% relative to toxin transfer by wild-type PA_{63}. More preferably, the decrease is at least 40%.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Brief Description of the Drawings

Fig. 1A shows the amphipathic sequences of the D2L2 loop of PA and the Gly-rich loop of α-hemolysin (aHL). Residues that form the hydrophobic face of the β barrel in a-hemolysin, or have been proposed to form the hydrophobic face of the PA_{63} pore, are underscored with a solid line. Residues that form the hydrophilic face in α-hemolysin, or have been proposed to form the hydrophilic face of the PA_{63} pore, are underscored with a dotted line.

Fig. 1B shows the proposed model for pore formation by PA_{63}. Following a low pH trigger, the D2L2 loops move to the base of the heptamer and combine to form a 14-stranded transmembrane β barrel.

Fig. 2 shows the effect of MTS-ET on N306C-induced macroscopic conductance. The current record (with the voltage held at +20 mV) begins 7 minutes after addition of trypsin-nicked PA N306C to a final concentration of 1.1 nM. At the arrow, MTS-ET was added trans to a concentration of 38 mM. After the initial artifactual increase in conductance due to the addition, the current is seen to decrease 6-fold within seconds.

Fig. 3 shows the reduction of conductance produced by trans MTS-ET as a function of location of Cys mutants within the D2L2 loop. Percent reduction was

calculated as [1-(IPA+MTS/IPA)] x 100, where IPA was the current immediately before MTS addition, and IPA+MTS was the lowest current observed within three minutes following addition of MTS-ET. Values are reported as the mean +/- standard error of 2-4 experiments. For A307C and I316C, MTS-ET-induced reduction in current was only seen in some experiments. Note the alternating pattern of reduction in conductance, except for the consecutive MTS-ET-responsive residues 312-315.

Fig. 4A shows the effect of MTS-ET on conductance of a single N306C channel. The trace begins after a single channel has opened with a conductance of 90 pS (at a holding potential of +50 mV). MTS-ET was then added to a concentration of 8 mM trans, and the current record was briefly obscured during stirring. One or more reactions occurred during stirring, since after the stirring was stopped the conductance of the channel was about half that before MTS-ET addition. The arrows indicate stepwise decreases in single channel conductance consistent with the reaction of MTS-ET with cysteines within the channel.

Fig. 4B shows that DTT reverses the MTS-ET effect. The trace begins five minutes after the final MTS-ET reaction was observed in Fig. 4A. DTT was added trans to a concentration of 1.2 mM, and the single channel conductance immediately increased during stirring. The arrows indicate further stepwise increases in single channel conductance, consistent with reduction of the mixed disulfides formed upon reaction of the cysteines with MTS-ET. At higher time resolution, the transition at the second arrow appears to be composed of 2-4 stepwise increases in conductance. (The last break in the record is 90 seconds.)

Fig. 5A shows that MTS-ET does not restrict the accessibility of cis Bu4N+ to its binding site. Current records (with voltage held at +20 mV) begin 3-5 minutes after addition of trypsin-nicked PA S325C. The arrows mark addition of Bu4N+. The addition of Bu4N+ cis to a concentration of 15 mM produced a 3.5-fold reduction in conductance.

Fig. 5B shows that MTS-ET does not restrict the accessibility of trans Bu4N+

to its binding site. Addition of Bu4N+ trans to a concentration of 500 mM produced a 2-fold reduction in conductance.

Fig. 5C shows that addition of Bu4N+ cis to a concentration of 15 mM to S325C channels previously modified with MTS-ET produced the same 3.5-fold fall in conductance as in Fig. 5A, despite the conductance having previously been substantially reduced due to modification of the channels' cysteines with MTS-ET.

Fig. 5D shows that addition of 500 mM Bu4N+ trans to S325C channels previously reacted with MTS-ET had essentially no effect on conductance; subsequent addition of 15 mM Bu4N+ cis produced its usual 3.5-fold fall in conductance. Thus, with the cysteines at positions 325 modified with MTS-ET, Bu4N+ added trans is prevented from reaching its binding site, whereas Bu4N+ added cis is not. These results argue that the Bu4N+ binding site lies cis to position 325.

Fig. 6 shows a model for the orientation of D2L2 within the membrane. The filled boxes indicate residues that are responsive to MTS-ET, based upon reduction of channel conductance. The open boxes indicate residues that show little or no effect upon MTS-ET addition. The pattern is consistent with each D2L2 contributing two antiparallel β strands to make a 14-stranded β barrel.

Detailed Description of the Invention

We have discovered that mutations to the pore forming molecule of pore-forming toxins creates a molecule which may prevent toxin translocation present during wild-type toxin exposure. Accordingly, the invention provides an antidote for use in treating exposure to pore forming toxins. The proteins of the invention also have immunogenic properties which may enhance host response to pore forming toxin exposure.

To test our antidote system we have used the B-moiety of anthrax toxin. In particular, we have mutated the PA_{63} polypeptide. Under low pH conditions, PA_{63} forms cation-selective channels in artificial membranes (Blaustein, R. O. et al., Proc

Natl Acad Sci U S A 86:2209-13, 1989; Koehler, T. M., & Collier, R. J., Mol. Microbiol. 5:1501-506, 1991) and in cell membranes (Milne, J. C. & Collier, R. J., Molec. Micro. 10:647-53, 1993). Purified PA_{63} was originally shown by electron microscopy to form ring-shaped heptamers (Milne, J. C. et al., J. Biol. Chem. 269: 20607-12, 1994), and recently the X-ray crystal structure of a heptameric, water soluble form of PA_{63} was determined (Petosa, C. et al., Nature 385;833-38,1997). Since this structure shows no regions of hydrophobicity that might mediate membrane insertion, this water soluble form structure may represent an intermediate, or prepore, in the insertion process.

The crystal structures of native PA and the PA_{63} prepore reveal the presence of a disordered, amphipathic loop (D2L2), which has alternating hydrophilic and hydrophobic residues reminiscent of the Gly-rich loop of the α-hemolysin (Fig. 1). This loop, which connects strands 2β2 and 2β3 within domain 2, projects outward from the side of domain 2 of each monomer within the water soluble heptamer (Petosa, C. et al., Nature 385;833-38,1997). A significant conformational rearrangement would be needed for this loop to participate in barrel formation, but a plausible mechanism for such a rearrangement has been proposed (Fig. 2) (Petosa, C. et al., Nature 385;833-38,1997).

Such a barrel formation has been reported for the bacterial pore-forming toxin α-hemolysin from Staphylococcus aureus. This toxin, which shows no sequence similarities to PA, forms a heptameric pore in membranes (Gouaux, J. E. et al., Proc Natl Acad Sci U S A 91:12828-31, 1994). The recently reported crystal structure of the pore indicates that the transmembrane motif consists of a porin-like 14-stranded β barrel formed from seven b hairpins (Song, L. et al., Science 274:1859-66, 1996). One b hairpin is contributed by each of the protomers, and each hairpin is in turn derived from a Gly-rich amphipathic loop in the monomeric water-soluble protein. The loop contains alternating hydrophilic and hydrophobic residues. Once assembled into the membrane-penetrant β barrel, the hydrophilic residues face the aqueous lumen

of the pore and the hydrophobic residues form the exterior, membrane-contiguous surface of the barrel.

To determine whether the 24 residues of the D2L2 loop of PA form a transmembrane β barrel channel similar to a-hemolysin, the substituted cysteine accessibility method was employed (Akabas, M. H. et al., Science 258:307-310, 1992). In this method, individual residues are replaced with cysteine and tested for accessibility to bilayer-impermeant methanethiosulfonate (MTS-ET) derivatives which specifically react with water-accessible sulfhydryls. Thus only solvent accessible cysteines would be expected to react with the reagent. Also, since the diameter of the PA_{63} channel has been estimated at 12 Å in channel blockage experiments (Finkelstein, A., Toxicology 87:29-41, 1994), whereas the diameter of MTS-ET is only 6 Å, only residues lining the channel have accessibility to the reagent (Akabas, M. H. et al., Science 258:307-310, 1992).

PA is ideally suited to this method, in that the native protein is devoid of cysteines. Should the cysteine of interest line the ion-conducting pathway, derivatization with the positively-charged reagent methanethiosulfonate ethyltrimethylammonium (MTS-ET) would introduce a positive charge within the cation-selective channel and likely result in reduction of channel conductance.

EXPERIMENTAL PROCEDURES

Materials.

MTS-ET bromide and sodium MTS-ES are available from Toronto Research Chemicals (North York, Ontario, Canada); Bu4N bromide, puriss grade, was obtained from Fluka (Buchs, Switzerland).

Construction and purification of cysteine mutants.

Wild type PA, containing a conservatively introduced Sal I site at 792 bp, was cloned into the Bam H1-Xho I sites of the *Escherichia coli* expression vector

pET22b+ from Novagen (Madison, WI), which directs for periplasmic expression. Site directed mutants were created from this template via two-step recombinant PCR using appropriate primers, and a 195 base pair Sal I-Eco RI fragment was subcloned back into the wild type vector. The ligation products were transformed into E. coli XL1-Blue from Stratagene (La Jolla, CA). The plasmid DNA was amplified, purified, and sequenced to confirm the presence of the mutation. Confirmed mutant plasmids were then transformed into the E. coli expression host BL21(DE3). Cultures were grown in Luria broth containing ampicillin at 37°C to an OD_{600} of 0.6-1.0, and protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (1 mM) for 3 hours at 30°C. Periplasmic proteins were extracted by first resuspending pelleted cells in 4 ml 20% sucrose, 5 mM EDTA, 150 mg/ml lysozyme, 20 mM Tris-HCl, pH 8.0, per gram of cells. After incubation on ice for 40 minutes, 80 ml of 1 M $MgCl_2$ per gram of cells was added. The mixture was centrifuged, and the resulting supernatant containing the desired protein was dialyzed overnight in buffer A (20 mM Tris, pH 8.0, 2 mM DTT, 1 mM EDTA). The protein was then purified by anion exchange chromatography (Q-sepharose followed by Mono Q) in buffer A. Occasionally, a further gel filtration step (Superdex 75) in buffer A containing 150 mM NaCl was required. Proteins were purified to 90% homogeneity, as judged by SDS-PAGE. Approximately 0.5 mg of purified protein was obtained from one liter of cells. Proteins were stored in 2 mM DTT at -80°C until use.

Trypsin activation of cysteine mutants.

Mutant proteins were diluted to 0.5 mg/ml in buffer A, and trypsin was added to a final concentration of 1 mg/ml. After incubation for 5 minutes at 37°C, soybean trypsin inhibitor was added to a final concentration of 10 mg/ml. Nicked proteins were stored on ice or at -80°C until use. Although the mutant proteins were stored in 2 mM DTT, it was found that maximal channel-forming activity and cysteine reactivity towards MTS-ET was obtained if fresh DTT, to an additional 2 to 5 mM,

was added to the nicked samples each day of use.

Macroscopic channel experiments.

Macroscopic conductance experiments were carried out at room temperature on planar lipid bilayers formed by the brush technique (Mueller et al., J Phys Chem 67: 534-535, 1963) across a 0.5 mm diameter hole in a Teflon partition separating two lucite chambers containing 3 ml of identical salt solutions (100 mM KCl, 1 mM EDTA, 10 mM dimethylglutaric acid, pH 6.6). The membrane-forming solution was 3% diphytanoylphosphatidylcholine in decane. After the membrane formed, trypsin-nicked mutant PA was added to the cis chamber to a final concentration of 30 pM to 3 nM (the final concentration of DTT in that chamber was typically 0.1-1 mM). Each chamber was stirred continuously throughout the experiment by small magnetic stir bars. After the mutant PA-induced current had stabilized, typically after 3-5 minutes, MTS-ET was added either to the cis chamber to a final concentration of 4 mM or to the trans chamber to a final concentration of 4-160 mM; these concentrations of MTS-ET had no effect on the current induced by nicked wild type PA. The effects of MTS-ET on mutant PA-induced conductance generally occurred over a period of tens of seconds. The percent decline of conductance produced by MTS-ET was calculated as [1-(IPA+MTS/IPA)] 100, where IPA was the current immediately before MTS-ET addition, and IPA+MTS was the lowest current observed within three minutes following addition of MTS-ET. Values are reported as the mean standard error of 2-4 experiments. In experiments involving Bu4N+, this reagent was added to a final concentration of 15 mM cis or 500 mM trans. All experiments were done under voltage clamp conditions (with the cis chamber held at +20 mV with relative to the trans chamber), using a single pair of Ag/AgCl electrodes that made electrical contact with the solutions in the chambers through 3 M KCl agar bridges. The current responses were filtered at 10 Hz and displayed on a Narco (Houston, TX) physiograph chart recorder.

Single channel experiments.

Planar bilayers were formed at room temperature from a 1% solution of diphytanoylphosphatidylcholine in hexane using a modification of the folded film method (Montal, M., Methods in Enzymology 32:545-554, 1974) across a 90-100 mm hole in a polystyrene cup (Wonderlin, W. F. et al., Biophys. J. 58:289-297,1990) as previously described (Silverman, J. A. et al., J. Membr. Biol. 137:17-28, 1994). The solutions both inside (0.5 ml) and outside (1 ml) the cup were the same as in the macroscopic experiments and could be stirred by small magnetic stir bars. Experiments were performed under voltage clamp conditions at +50 mV cis. In a typical experiment, nicked mutant PA was added, after the membrane formed, to a final concentration of 150 pM-16 nM to the outside chamber (cis), and the resulting current responses were monitored on a chart recorder and recorded on digital tape as previously described (Silverman, J. A. et al., J. Membr. Biol. 137:17-28, 1994). After a channel appeared, MTS-ET was added to the trans chamber to a concentration of ~1 mM. Solutions were stirred as necessary. For the DTT reversal experiments, DTT was subsequently added to the trans chamber to a final concentration of ~1 mM.

RESULTS

A series of 24 mutations, each replacing an individual D2L2 residue with cysteine, was generated via recombinant PCR. DNA fragments encoding the desired mutation were subcloned into wild type PA within the pET22b+ vector, and the mutant proteins were expressed in *E. coli*, harvested from the periplasm, and purified via anion exchange and gel filtration chromatography to 90% homogeneity.

PA is cleaved *in vitro* into its 20 kDa and 63 kDa fragments by treatment with trypsin. When trypsin-activated PA is added to artificial lipid bilayers and the pH is reduced below pH 7 in the presence of 1 mM EDTA, cation-selective channels are formed, which are identical to those formed by purified PA_{63} (Blaustein, R. O. et al.,

Proc Natl Acad Sci U S A 86:2209-13, 1989). All of the D2L2 cysteine mutant proteins formed channels in planar lipid bilayers as readily as wild-type, provided they had been activated with trypsin and fully reduced.

Macroscopic conductance measurements.

For macroscopic conductance experiments, in which the collective conductance of hundreds to thousands of channels is measured, sub-nanomolar to nanomolar concentrations of trypsin-activated mutant were added to painted diphytanoyl-phosphatidylcholine bilayer membranes held at a voltage of + 20 mV at pH 6.6. Once the current had more or less plateaued, MTS-ET was added to the trans compartment (the compartment opposite that to which PA was added), and its effect on macroscopic current was observed. The positively-charged methanethiosulfonate reagent MTS-ET was chosen because of the known cation selectivity of PA_{63} channels. For certain mutants, an immediate and large decline in current was observed, whereas for others no decline was detected, even after several minutes.

Fig. 2 shows a typical trace for a MTS-ET-responsive mutant. Addition of 38 mM MTS-ET trans to channels formed by the N306C mutant reduced macroscopic current nearly 6-fold within seconds. Subsequent trans addition of 4 mM DTT caused a reversal of the effect. The current induced by most other MTS-ET-responsive mutants was also maximally reduced within seconds following MTS-ET addition. With some mutants, the decrease was more gradual, but in all cases was essentially complete within three minutes. The reduced current persisted with most mutants for the duration of the experiment. In some experiments, the decline in G317C- and S319C-induced current produced by MTS-ET added trans was followed by a linear rise in current to levels exceeding that which was observed before MTS-ET addition. The reasons for this effect are unknown, but, without wishing to be bound by the following, we believe it is conceivable that reaction of MTS-ET at these sites catalyzes disulfide formation between monomers which could alter channel structure

to a state of higher conductance. Neither MTS-ET nor DTT, at the concentrations used in these experiments, showed any effect on wild type channels.

Fig. 3 shows the maximal reduction of macroscopic current attained by each mutant within the first three minutes following trans-addition of MTS-ET. Within each of two stretches of D2L2 (E302C-A311C and I316C-S325C), alternating positions displayed a reduction of current following the addition of MTS-ET. All hydrophilic positions within these stretches were responsive to MTS-ET, whereas all hydrophobic positions displayed little to no MTS-ET effect. (The only exceptions were positions A307C and I316C, where a slow effect was seen in some experiments.) These stretches were bridged by a region of consecutive MTS-ET-responsive residues, from S312C-D315C. The pattern of current reduction was identical when MTS-ET (~4 mM) was added to the cis side. When methanethiosulfonate ethylsulfate (MTS-ES, ~100 mM), which adds a negatively charged sulfate group to a reactive cysteine, was added in place of MTS-ET, the responses of the mutant channels were more variable: reduction of current was seen at some positions, and increase of current was seen at other positions, including all sites where a negatively-charged native residue (E302, E308, and D315) had been replaced. This variability is not surprising, considering introduction of MTS-ES increases negative charge within the cation-selective channel while also introducing steric bulk which may hinder ion flow. Despite this variability, a response to MTS-ES occurred only at positions that were also responsive to MTS-ET. These results, as a whole, support the proposed model of insertion of each D2L2 as a β hairpin.

Single-channel measurements.

If the proposed model of channel formation is correct, single-channel measurements of MTS-ET-responsive mutants might permit one to resolve individual reactions between MTS-ET and each of the 7 single cysteine residues within the heptameric pore. We performed single-channel measurements with two mutants,

N306C and H304C. In a typical experiment, trypsin-activated mutant PA was added to a diphytanoylphosphatidylcholine membrane separating identical salt solutions (100 mM KCl) at pH 6.6, and generally a single channel opened within 30 minutes. The conductance of the N306C and H304C channels was the same as that of wild-type channels (~100 pS at an applied voltage of +50 mV) (Blaustein, R. O. et al., J Gen Physiol 96:921-42, 1990). MTS-ET was then added to the trans compartment, the solutions were stirred briefly, and the effect on conductance was observed. With single N306C channels, stepwise jumps to lower conductance states were seen following addition of MTS-ET, consistent with multiple reactions within a multimeric channel (Fig. 4A). Furthermore, subsequent addition of DTT reversed the MTS-ET effect: multiple stepwise increases in channel conductance, consistent with DTT-mediated reduction of the mixed disulfide between the cysteines and ethyltrimethylammonium (the product of the MTS-ET reactions), were seen until original channel conductance was regained (Fig. 4B). Similar effects were seen when MTS-ET was added to single channels formed by H304C.

The pattern of conductance inhibition by MTS-ET was similar in all single-channel experiments: a decrease in conductance occurred during or immediately following addition of MTS-ET, and additional jumps to lower conductance states occurred afterwards. Up to five transitions to lower conductance states were resolved within some single channels. The state of lowest conductance ranged from 28 pS to 12 pS (72%-88% reduction), depending upon the experiment, and the magnitudes of individual transitions varied from experiment to experiment. In the putative heptameric channel, 19 spatial combinations would be allowed for derivatization of 1-7 cysteines (Braga, O. et al., Chem Biol 4:497-505, 1997). (For instance, the second MTS-ET could modify cysteines at any of three different locations relative to the first -- an adjacent residue, or one or two monomers removed.) Each configuration may result in a different reduction of channel conductance through a combination of steric and electrostatic effects.

In the course of performing the experiments described above, the observation was made that reaction with MTS-ET and reversal by DTT could apparently occur from the trans compartment while the channel was temporarily closed. For example, during single channel experiments with N306C, a channel often transiently closed for a few seconds. In some cases, when the channel closed in the presence of trans MTS-ET, it re-opened to a lower conductance state. Similarly, following the addition of DTT, a closed channel sometimes reopened to a higher conductance state. Such results imply that D2L2 forms a channel which is gated at a site cis relative to position 306.

Bu4N+ accessibility.

Symmetric quaternary ammonium ions ranging in size from tetramethyl to tetraheptyl have been shown to block PA_{63} channels in a voltage dependent manner (Blaustein, R. O., & Finkelstein, A., J Gen Physiol 96:905-19, 1990). In studies with Bu4N+, an approximately 40-fold higher trans concentration at +20 mV was required to yield the same effect as a given cis concentration at that voltage. Macroscopic and single channel analyses led to a two-barrier single-well energy barrier model for binding of Bu4N+ within the PA_{63} channel (Blaustein, R. O. et al., J Gen Physiol 96:921-42, 1990; Blaustein, R. O., & Finkelstein, A., J Gen Physiol 96:905-19, 1990). To test whether one of the residues of D2L2 participates in binding Bu4N+, we screened each mutant for the ability to yield a Bu4N+ blocking effect. In each mutant, as in wild-type channels, a rapid 2-5 fold reduction of macroscopic current was seen when Bu4N+ was added to the cis compartment to a concentration of 15 mM (e.g. Fig. 5A). The ability of each mutation to yield a normal Bu4N+ blocking effect suggests that the binding site does not involve the D2L2 residues and, therefore, lies elsewhere in the protein.

To investigate if the Bu4N+ binding site is located cis with respect to the inserted D2L2, we first reacted channels of S325C, the site proposed to be located

farthest cis, with MTS-ET and then determined whether subsequent cis or trans addition of Bu4N+ had its usual effect on conductance. We reasoned that electrostatic repulsion from the ethyltrimethylammonium ion bound to reacted cysteines within the channel would inhibit flow of Bu4N+ ions past the derivatized region and thereby inhibit its access to its binding site, if the site lay beyond the derivatized region. As seen in Fig. 5C, prior reaction with MTS-ET at residue S325C did not prevent subsequent cis 15 mM Bu4N+ from producing its usual rapid 3.5-fold fall in conductance, seen in Fig. 5A, even though the conductance was already substantially reduced. By itself, this result strongly suggests that the Bu4N+ binding site lies cis relative to D2L2. As further confirmation, prior reaction of S325C with MTS-ET prevented trans 500 M Bu4N+ from producing its usual two-fold fall in conductance (compare Fig. 5D to 5B). One would expect such a result if the Bu4N+ binding site lies cis relative to residue 325, as the ethyltrimethylammonium ion bound to reacted cysteines at that site would inhibit trans Bu4N+ from reaching its binding site. These results strongly suggest that the Bu4N+ binding site is located cis relative to D2L2, presumably within the putative globular cap region of the pore.

DISCUSSION

Although many bacterial toxins act by modifying cytosolic substrates within the mammalian cell, the mechanism by which the enzymic moiety of a toxin crosses a membrane has not been understood. Nonetheless, this is a step which is essential to the ultimate death of the cell from toxin exposure. In some A-B toxins, such as diphtheria and anthrax toxins, the B moiety inserts into membranes under translocation conditions to form a pore which may serve as a conduit for A-chain translocation. To probe this hypothesis effectively, it is essential to understand the structure of the pore in detail. The recently solved crystal structure of the heptameric PA_{63} prepore of anthrax toxin has provided a detailed structure of an intermediate in pore formation (Petosa, C. et al., Nature 385:833-38, 1997). We have now tested a

model of prepore-to-pore conversion involving formation of a transmembrane 14-stranded β barrel from the D2L2 loop of domain 2 of PA. The results of the cysteine assessibility experiments represent strong evidence in favor of the model.

The probe used for assessing cysteine assessibility, MTS-ET, has been shown to be bilayer-impermeant by liposome leakage and excised patch experiments (Holmgren, M. et al., Neuropharmacology 35:797-804, 1996), and derivatization of cysteines by methanethiosulfonate reagents has been shown to occur 10 orders of magnitude faster when cysteine is in the hydrated thiolate anion form rather than the sulfhydryl form (Roberts, D. D. et al., Biochemistry 25:5595-5601, 1986). Thus, only solvent accessible cysteines would be expected to react with the reagent. Also, since the diameter of the PA_{63} channel has been estimated at 12 Å in channel blockage experiments (Finkelstein, A., Toxicology 87:29-41, 1994), whereas the diameter of MTS-ET is only 6 Å (Akabas, M. H. et al., Science 258:307-310, 1992), we expected residues lining the channel to be accessible to the reagent. Finally, if a cysteine lining the ion conducting pathway reacted with MTS-ET, the introduction of positive charge would be likely to inhibit ion conductance within the cation-selective PA_{63} channel.

The pattern of inhibition of macroscopic conductance by MTS-ET was strikingly close to that predicted by the model (Fig. 6). Alternating residues within each of two stretches (302-311, 316-325) that had been predicted to form the antiparallel strands of the β barrel showed different effects following addition of the reagent. Residues predicted to lie on the polar, luminal face of the barrel showed strong reduction of conductance by MTS-ET, whereas those predicted to lie on the apolar, membrane-contiguous face showed little to no reduction. The lack of effect in the latter case most likely reflects inaccessibility to the reagent.

The two regions of alternating accessibility were bridged by a short segment of fully accessible residues (residues 312-315) that encompass the residues which had been predicted to form the β turn of the putative hairpin (Fig. 1A). Two aromatic residues in this region, F313 and F314, presumably border the membrane/water

interface at the trans face of the bilayer. Aromatic residues have been shown to lie at the lipid/water interface and girdle β barrels formed by porins, as seen in the crystal structure of a porin from Rhodopseudomonas blastica (Kreusch, A. et al., Protein Sci. 3:58-63, 1994). The β turn region within D2L2 of protective antigen is presumably different than that adopted by the Staphylococcus aureus α-hemolysin: in PA_{63}, the i and i+3 residues of the turn need to lie on the same face of the hairpin, while in the a-hemolysin, i and i+3 apparently lie on opposite sides of the barrel. Only two minor anomalies in the overall pattern of inhibition were observed: in some experiments, a weak effect of MTS-ET was detected at A307C and I316C. This may reflect greater flexibility of the β structure at these locations.

The absence of an MTS-ET effect at hydrophobic positions in D2L2 does not, of course, prove that these residues face the interior of the bilayer. While not intending to be bound by the following assertions, it is conceivable that one or more of these positions are in contact with the channel, but their reactivity is inhibited by other mechanisms; alternatively reactions may occur but have no effect on conductance at these positions. It is also possible that these residues form the exterior surface of a channel segment which does not penetrate the membrane. The consistent pattern to the MTS-ET effects, however, makes these possibilities unlikely.

Also consistent with the proposed model are the results of single-channel measurements, showing multiple step-wise conductance changes in response to MTS-ET at positions 306 and 304. The stepwise changes were reversible with DTT and were consistent with single reaction events involving individual cysteines in the channel. The data imply that the channel is comprised of an oligomeric structure, correlating with the heptameric, ring-shaped structure of PA_{63} observed by electron microscopy and X-ray crystallography (Petosa, C. et al., Nature 385:833-38, 1997; Milne, J. C. et al., J. Biol. Chem. 269:20607-20612, 1994). We resolved up to five MTS reactions per channel, but this probably does not reflect the maximum number of protomeric cysteines lining the pore. Because each successive MTS-ET reaction

lowers conductance by introducing greater electrostatic repulsion and steric constraints within the channel, derivatization of all cysteines may not have been possible within the time frame of a normal experiment.

The variation in stepwise changes in conductance from experiment to experiment may reflect the large number of possible configurations of reacted cysteines within the channel. Up to 19 configurations of 1-7 reacted cysteines within a heptamer are theoretically possible (Braga, O. et al., Chem Biol 4:497-505, 1997), and each configuration may have a unique effect on conductance resulting from steric and electrostatic effects. Multiple MTS reactions within a single channel have also been observed in analysis of the ryanodine receptor channel (Quinn, K. E., & Ehrlich, B. E., J Gen Physiol 109:255-64, 1997), where four discrete subconductance states of 3/4, 1/2, 1/4, and 0-fold of the unreacted channel were seen. In contrast, single channel analysis of the effects of MTS reagents on cysteine-substituted channels formed by the diphtheria toxin T domain has revealed only one stepwise decrease in conductance per channel, consistent with a single MTS-ET reaction within a monomeric channel (Huynh, P. D. et al., J. Gen. Physiol. 110:229-242, 1997).

According to the proposed model for channel formation by PA_{63}, the D2L2 loops move to the base of the heptameric prepore, where they form a membrane-inserted β barrel (Petosa, C. et al., Nature 385:833-8, 1997). The globular domains of the heptameric ring would then extend the pore above the cis leaflet of the membrane, as seen with the Staphylococcus aureus a-hemolysin pore. We have found that the channel blocker Bu4N+ is prevented from reaching its binding site when added to the trans (but not the cis) compartment of S325C channels pre-derivatized with MTS-ET. This indicates that the binding site for Bu4N+ is located cis relative to the inserted D2L2 loops. The lumen of the prepore is predominately negatively charged (Petosa, C. et al., Nature 385:833-8, 1997), and assuming there is little change in that lumen during the prepore-to-pore conversion, it represents a likely locus for binding of the Bu4N+ ion. In addition, the ability of trans MTS-ET and DTT to react

within the channel in its closed state suggests that the gate lies cis to the membrane-inserted region defined here, and this gate may also reside within the globular cap of the pore.

Within the heptameric prepore, the D2L2 loops are located midway up the globular domains and are flanked by the 2β2 and 2β3 strands of domain 2. For D2L2 to move to the base of the prepore to form the β barrel, it has been proposed that β and 2b3 tear away from the body of domain 2 under the influence of low pH (Petosa, C. et al., Nature 385:833-8, 1997). Residues of the 2β2 and 2β3 strands may also contribute to the channel by forming an extension of the β barrel formed by D2L2, and this extension may serve as a link between the membrane-inserted and globular regions. This extension represents an alternative locus for Bu4N+ and/or channel gating sites.

Pore-forming bacterial toxins undergo a variety of conformational changes in the transition from a secreted hydrophilic protein to one capable of penetrating a hydrophobic membrane. Such transitions may involve the creation of entirely new surfaces, such as the β barrel for anthrax protective antigen, as presently described, and for the membrane-associated α-hemolysin (Song, L. et al., Science 274:1859-66, 1996).

Here we show that disrupting these surfaces can prevent effective pore formation. Therefore, if a pore-forming toxin protein had a deletion of the amino acid sequence that forms the transmembrane pore, such a protein could to neither effectively form a transmembrane pore nor translocate toxin into a host cell. Such a mutant could also function as a dominant mutant, effectively inhibiting the translocation of wild type toxin by forming hybrid heptamers with the wild-type toxin proteins and interfering with normal pore formation.

Administration of these mutant proteins could be effective both prophylatically and in the treatment of a patient exposed to a pore-forming toxin, or the bacteria producing the toxin. Prophylactic administration could also have the added advantage

of inducing the production of antibodies in the patient directed against the wild-type toxin.

Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth. All references are herein incorporated by reference.

What is claimed is:

Claims

1. A purified protein comprising a mutant pore-forming toxin, wherein the toxin comprises a mutation in an amino acid that forms the transmembrane pore of said toxin.

2. The protein of claim 1, wherein said mutation is genetically engineered, isa deletion, is a missense mutation, is located at the hydrophilic face of a transmembrane pore, reduces the toxicity of a wild-type pore-forming toxin, inhibits the translocation of a pore-forming toxin across said transmembrane pore, or decreases conductance measured at said transmembrane pore.

3. The protein of claim 1, wherein said toxin is a mutant B-moiety of an A-B toxin, a binary toxin, an anthrax toxin protective antigen (PA), or the C-terminal 63 kDa tryptic fragment (PA_{63}).

4. The protein of claim 3, wherein said toxin is a mutant B-moiety or said mutation is located in the amino acid sequence of the D2L2 loop of said B-moiety.

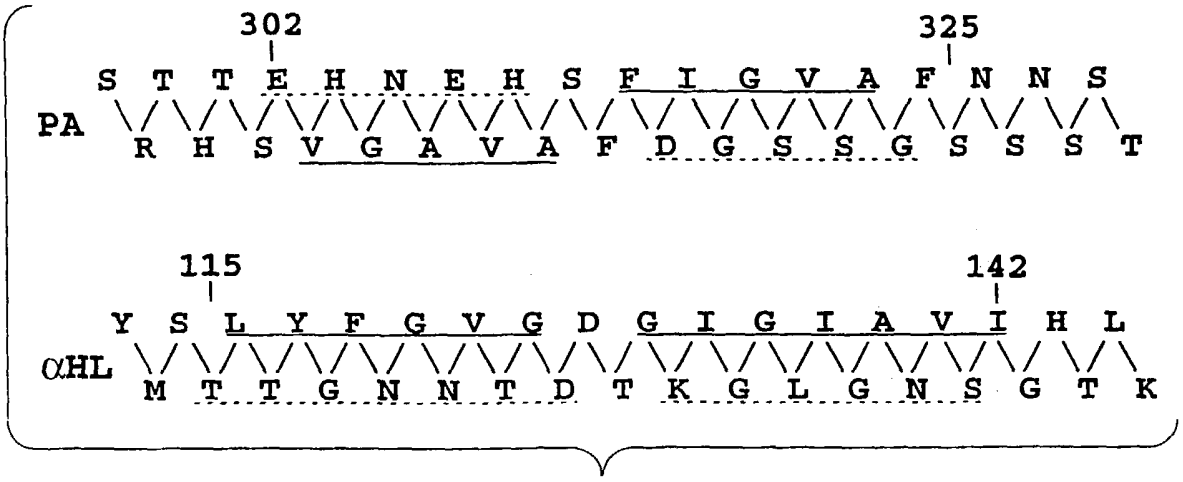
5. The protein of claim 3, wherein said toxin is an anthrax toxin protective antigen (PA), the C-terminal 63 kDa tryptic fragment (PA_{63}), and said mutated amino acid residue is E302, H304, N306, E308, H310, S312, F313, F314, D315, G317, S319, S321, G323, or S325 of the PA or PA_{63} D2L2 loop.

6. A substantially pure nucleic acid encoding the amino acid sequence of a mutant pore-forming toxin, wherein said toxin has a mutation in an amino acid that forms the transmembrane pore of said toxin.

7. A method of decreasing toxicity of a pore-forming toxin, said method comprising administering a protein comprising a mutant pore-forming toxin, wherein said toxin comprises a mutation in an amino acid sequence that forms the transmembrane pore of said toxin, in a dose sufficient to inhibit translocation of a pore-forming toxin.

8. The method of claim 7, wherein said mutant pore-forming toxin functions as a dominant mutant to inhibit translocation of a wild-type pore-forming toxin and said mutant pore-forming toxin, or is administered prophylactically.

9. A method of decreasing toxicity of a pore-forming toxin, said method comprising administering a protein comprising a mutant pore-forming toxin, wherein said toxin comprises a mutation in an amino acid sequence that forms the transmembrane pore of said toxin and generates antibody production directed against a wild-type sequence of said pore-forming toxin.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325
S T T E H N E H S F I G V A F N N S
PA
R H S V G A V A F D G S S G S S S T
115
142
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αHL
M T T G N N T D T K G L G N S G T K

Fig. 1A

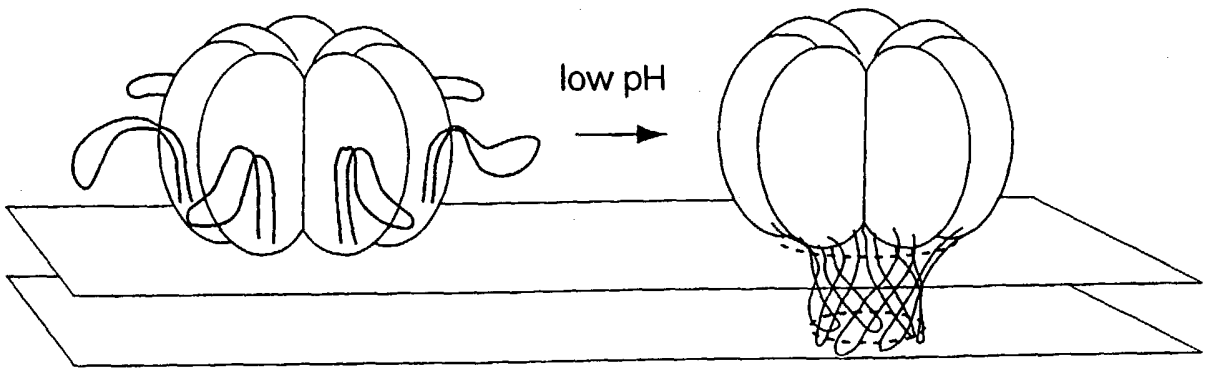

low pH

Fig. 1B

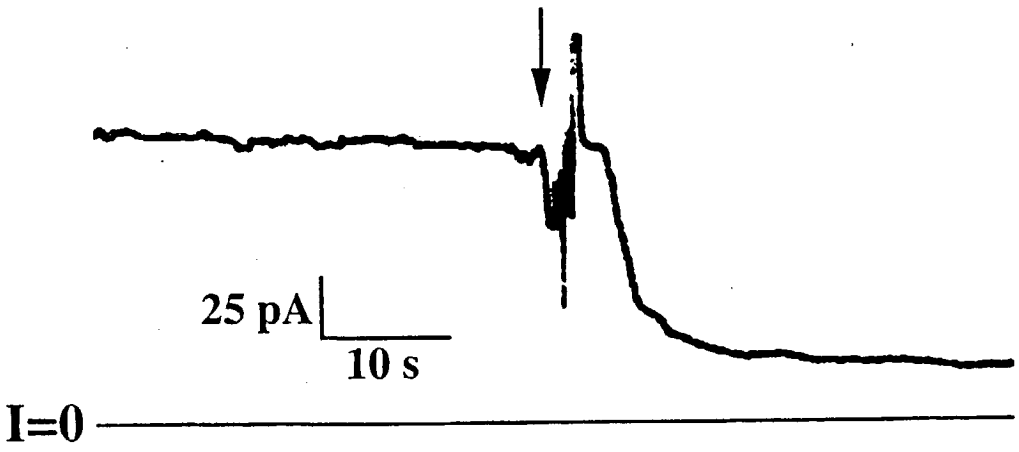
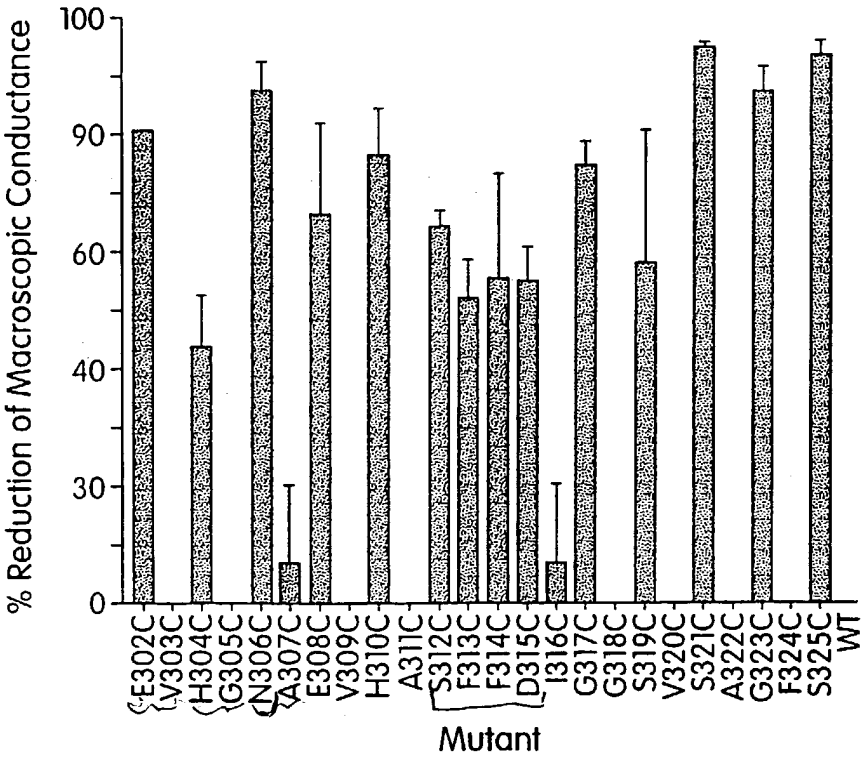


Fig. 2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100
90
60
40
30
0
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V303C
H304C
G305C
N306C
A307C
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H310C
A311C
S312C
F313C
F314C
D315C
I316C
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G323C
F324C
S325C
WT
Mutant

Fig. 3

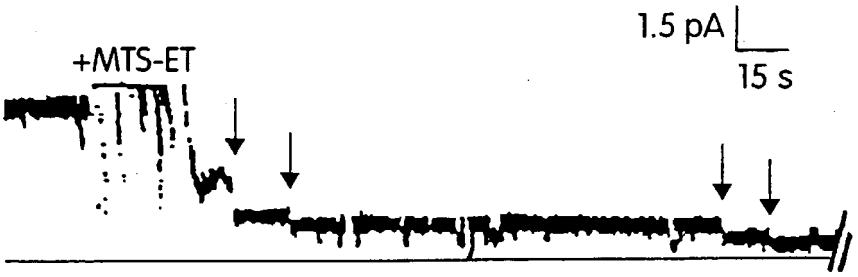


Fig. 4A

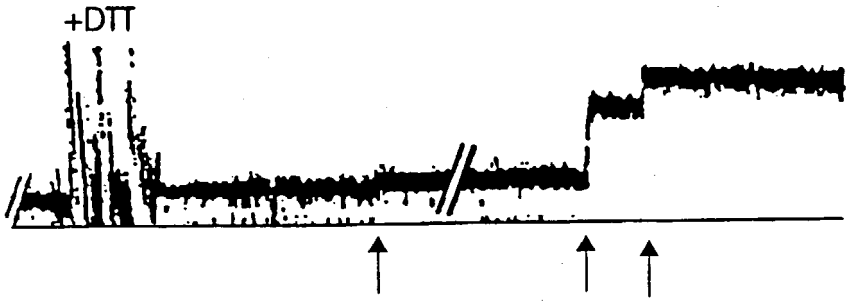


Fig. 4B

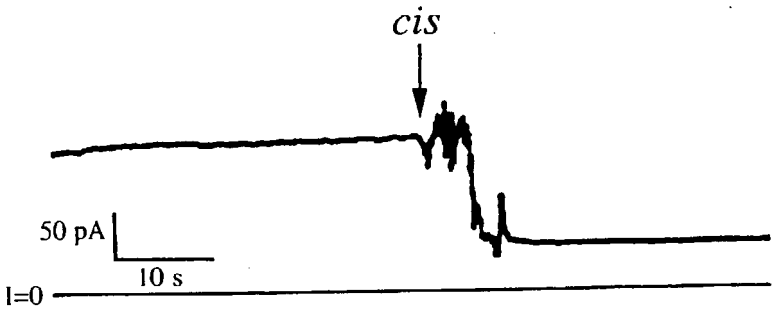


Fig. 5A

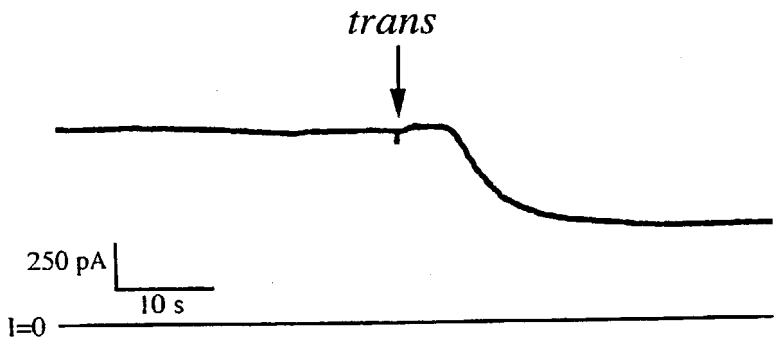


Fig. 5B

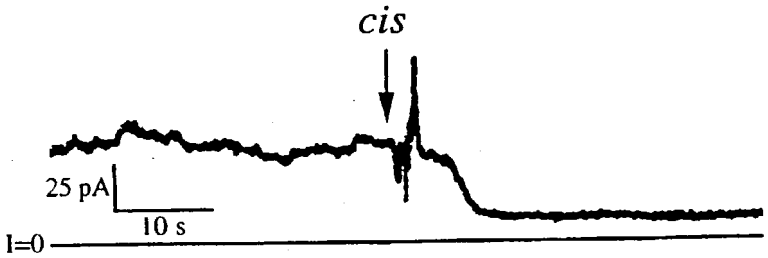


Fig. 5C

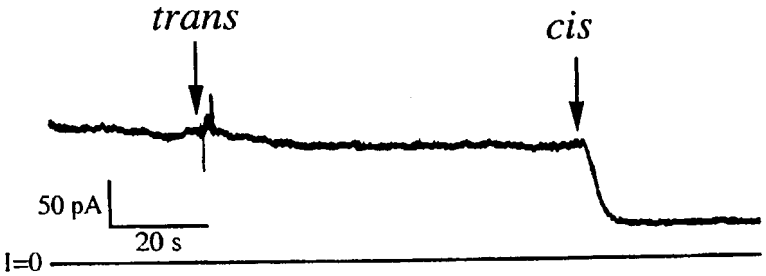
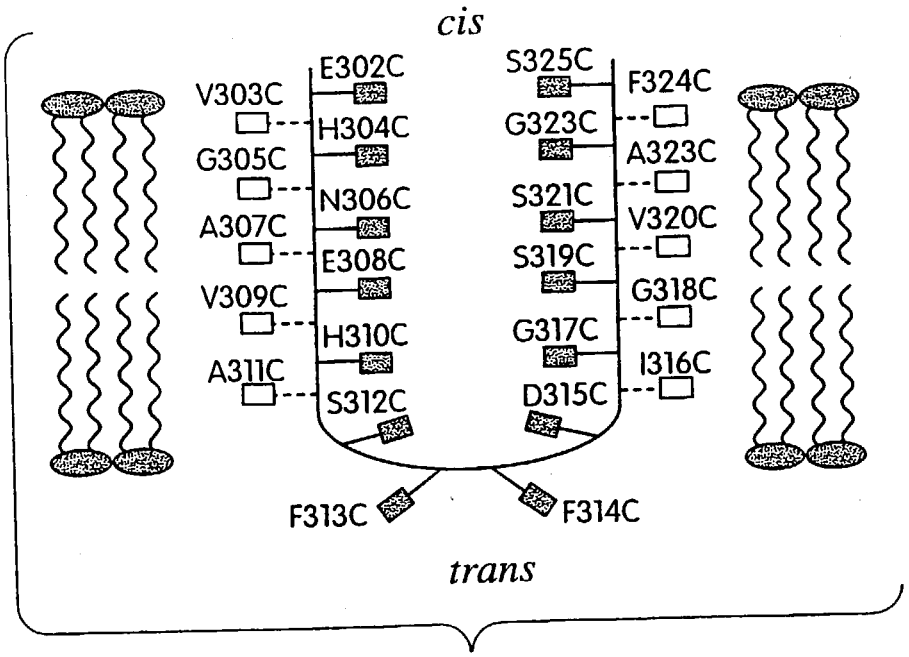


Fig. 5D


cis
E302C
V303C
H304C
G305C
N306C
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H310C
A311C
S312C
F313C
F314C
S325C
F324C
G323C
A323C
S321C
V320C
S319C
G318C
G317C
I316C
D315C
trans

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00, C12Q 1/48, C12P 21/06, C12N 13/00

US CL : 530/350; 435/15, 69.1, 173.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 435/15, 69.1, 173.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN (PORE-FORMING TOXIN#, ANTIBOD?, TOXICIT?, MUAT?, ANTHRAX TOXIN#)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WALKER et al. A Pore-Forming Protein with a Protease- Activated Trigger. Protein Engineering. 1994, Vol. 7, No 1, pages 91-97, especially page 91.	1, 3, 6 ------- 4-5
X	NARAT et al. The Humoral and Cellular Immune Response to a Lipid Attenuated Pore-Forming Toxin From The Sea Anemone Actinia Equina L. Toxion. 1994, Vol. 32, No. 1, pages 65-71, entire document.	1-3, 7-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the [illegible] cannot be considered to involve an [illegible] the document is combined with one or more [illegible], such combination being obvious to a person [illegible]
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the [illegible]y

Date of the actual completion of the international search

08 APRIL 1999

Date of mailing of the inte[illegible]ch report

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03457

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WALEV et al. Transmembrane Beta-Barrel of Staphylococcal Alpha-Toxin Forms in Sensitive but not in Resistent Cells. Proc. Natl. Acad. Sci. USA. October 1997, Vol. 934, pages 11607-11611, especially page 11607.	1-3, 6-8 -------- 4-5
Y	US 5,591,631 A (LEPPLA et al.) 07 January 1997, col. 3, lines 29-55; col. 7-8.	4-9